Glutathione Protects the Rat Liver Against Reperfusion Injury After Prolonged Warm Ischemia

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Objective: To evaluate the potential of postischemic intravenous infusion of the endogenous antioxidant glutathione (GSH) to protect the liver from reperfusion injury following prolonged warm ischemia.

Background Data: The release of reactive oxygen species (ROS) by activated Kupffer cells (KC) and leukocytes causes reperfusion injury of the liver after warm ischemia. Therefore, safe and cost-effective antioxidant strategies would appear a promising approach to prevent hepatic reperfusion injury during liver resection, but need to be developed.

Methods: Livers of male Lewis rats were subjected to 60, 90, or 120 minutes of normothermic ischemia. During a 120 minutes reperfusion period either GSH (50, 100 or 200 μmol/h/kg; n= 6–8) or saline (n= 8) was continuously administered via the jugular vein.

Results: Postischemic GSH treatment significantly prevented necrotic injury to hepatocytes as indicated by a 50–60% reduction of serum ALT and AST. After 1 hour of ischemia and 2 hours of reperfusion apoptotic hepatocytes were rare (0.50 ± 0.10%; mean ± SD) and not different in GSH-treated animals (0.65 ± 0.20%). GSH (200 μmol GSH/h/kg) improved survival following 2 hours of ischemia (6 of 9 versus 3 of 9 rats; $P < 0.05$). Intravital fluorescence microscopy revealed a nearly complete restoration of sinusoidal blood flow. This was paralleled by a reduction of leukocyte adherence to sinusoids and postsinusoidal venules. Intravenous GSH administration resulted in a 10- to 40-fold increase of plasma GSH levels, whereas intracellular GSH contents were unaffected. Plasma concentrations of oxidized glutathione (GSSG) increased up to 5-fold in GSH-treated animals suggesting counteraction of the vascular oxidant stress produced by activated KC.

Conclusions: Intravenous GSH administration during reperfusion of ischemic livers prevents reperfusion injury in rats. Because GSH is well tolerable also in man, this novel approach could be introduced to human liver surgery.

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Temporary clamping of the portal triad, ie, inflow occlusion by the Pringle maneuver,[1] is a common strategy to minimize bleeding during hepatic resection. However, portal triad clamping can induce relevant ischemia reperfusion injury (IRI) and failure of the remnant liver.[2–7] Because prolonged ischemia is frequently unavoidable to achieve radical tumor resection,[2] therapeutic strategies to minimize IRI are needed.

The precise sequence of events leading to IRI following warm liver ischemia has not been completely elucidated. However, there is substantial evidence that activation of Kupffer cells (KC), the generation of reactive oxygen species, (ROS) and disturbances of the hepatic microcirculation contribute to reperfusion injury.[6,8–12] During reperfusion activated KC produce mediators of inflammation and release ROS into the sinusoidal space.[6,12] The resulting vascular oxidant stress has been discovered as potential mechanism of hepatic perfusion failure.[13–16] ROS can activate redox-sensitive transcription factors, thereby activating proinflammatory genes and adding to the hepatic damage.[17,18] Furthermore, ROS may contribute to the activation of calcium–dependent proteases (calpains)[19] which mediate several intracellular processes resulting in necrotic or apoptotic cell death.[20,21]

Thus, ROS can be considered as signal molecules, which trigger several pivotal mechanisms of reperfusion injury. Consequently, antioxidant strategies appear a promising approach to prevent hepatic reperfusion injury.[10] Recent studies investigated the therapeutic potential of the *endogenous* antioxidant *glutathione (GSH),* in particular since it has a low toxicity in humans[22] and is cost-effective. Earlier studies indicated that GSH is able to react spontaneously with nearly all oxidants formed during inflammation.[23–25] Subsequent studies demonstrated that GSH released through the

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GSH transporter of hepatocytes may act as an endogenous defense system against KC–derived ROS, thereby protecting the hepatic vasculature from damage by KC.[8] The hepatic GSH content which can decrease rapidly during preoperative starvation is pivotal for this defense system.[26] Thus, any intervention increasing hepatic or plasma GSH levels should convey protection against reperfusion injury. Compounds with potential therapeutic value include N–acetylcysteine,[27] methionine,[28] glutathione esters[29] but also GSH itself. As shown recently in vitro, treatment of *cold* preserved livers with 2 or 4 mM GSH upon reperfusion prevented cell damage to hepatocytes in the model of cell-free rat liver perfusion.[30]

Therefore, the purpose of this study was to test the hypothesis that intravenous administration of GSH protects the rat liver against reperfusion injury after prolonged *warm* ischemia in vivo. In particular, the aims of the current study were: to investigate a potential influence of intravenously applied GSH on necrotic and apoptotic cell death, disturbances of the hepatic microcirculation and animal survival and to determine the functional significance of changes of the extra- and intracellular antioxidant capacity.

METHODS

Surgical Procedure

Male Lewis rats (250 g) were purchased from Charles River Wiga (Sulzfeld, Germany) and housed in a temperature- and humidity-controlled room under a constant 12-hour light/dark cycle. Animals had free access to water and rat chow (standard diet, Altromin, Germany). All experiments were performed with rats fasted 12 hours prior to operations. All studies were performed with the permission of the Government Authorities and in accordance with the German Legislation on Laboratory Animal Experiments.

Surgery was performed under spontaneous ether inhalation. Arterial blood pressure was continuously monitored via a carotid catheter. Another catheter was inserted into the jugular vein for substitution of plasma volume and injection of fluorescent compounds. Body temperature was kept between 36.5°C and 37.5°C by means of a heating pad. After laparotomy, ischemia of the left lateral and left median liver lobes was induced.[31,32] Reflow was initiated by removal of microclips, which selectively clamped branches of the portal vein and hepatic artery. Blood samples (500 μL) obtained at 15, 30 and 120 minutes upon reperfusion were replaced by the same volume of saline. Thereafter, experiments were terminated and the liver weight was determined.

Experimental Design

For evaluation of the early reperfusion injury, 2 control groups were compared with a total of 4 intervention groups and one sham-operated group, respectively.

Sham group. animals underwent laparotomy and intravital microscopy without hepatic ischemia (n=6).

Control group. livers were subjected to partial ischemia at 37°C for 60 (n=8) or 90 (n=8) minutes and subsequent reperfusion for 120 minutes.

Treatment groups. livers were subjected to 60 minutes of partial ischemia. Intravenous administration via the jugular vein of either 50 μmol GSH/h/kg (Tationil 600, Boehringer Mannheim/Italy) (n=8), 100 μmo/h/kg (n=8) or 200 μmol GSH/h/kg (n=6) was started 20 minutes before reperfusion and continued until the end of the 120 minutes reperfusion period. Another group (n=6) was subjected to 90 minutes of warm ischemia with intravenous administration of 200 μmol GSH /h/kg during reperfusion as mentioned above.

Saline GSH stock solutions (0.24, 0.48 and 0.96 M) adjusted to a pH of 6.5 were administered by continuous infusion (2 mL/h) using a microinfusion pump (SP 100i, WPI, Aston, UK).

Animal Survival

Experiments to assess animal survival were performed by resecting the nonischemic liver lobes at the end of a 2 hour period of warm ischemia of the left and median liver lobes.[33] Following reperfusion with or without intravenous infusion of 200 μmol GSH /h/kg (each n=9) animals were fed ad libitum and observed for 30 days.

In Vivo Fluorescence Microscopy (IVM)

IVM studies were performed 30 minutes after starting reperfusion at stable hemodynamic conditions, using a modified Leitz-Orthoplan microscope combined with epi-illumination technique.[34–36] For visualization of the microcirculation, sodium fluorescein (1μmol/kg; Merck AG, Darmstadt, Germany) and rhodamin 6G (0.1 μmol/kg; Merck AG, Darmstadt, Germany) were injected intravenously for fluorescent staining of hepatocytes and leukocytes, respectively. Quantification of microhemodynamic parameters was performed offline by frame-to-frame analysis of the videotaped images in a blinded fashion. Lobular perfusion and leukocyte adherence were analyzed by scanning a region of 10 randomly selected acinar areas and postsinusoidal venules, respectively. Nonperfused sinusoids were estimated by counting the number of continuously perfused and nonperfused sinusoids and was expressed as the percentage of all sinusoids visible in a predefined area. Permanent adherence (*sticking*) of leukocytes to the sinusoidal endothelium was quantified by counting the number of permanently attached cells (at least for 20 seconds) within periportal, midzonal and pericentral segments of sinusoids as reported previously.[37] Leukocytes sticking in postsinusoidal venules were determined by the quantity of cells attached for at least 20 seconds to the surface of postsinusoidal venules. Furthermore, temporary adherence of leukocytes (*rolling*) was assessed as the number of transiently attached leukocytes along the endothelial surface of

postsinusoidal venules during an observation period of 20 seconds.

Histology and TUNEL Assay

Formalin-fixed tissue samples were embedded in paraffin, and 5- μm sections were cut. Sections were either stained with hematoxylin and eosin (HE) or with the terminal desoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) assay (ApopTag Peroxidase In Situ Apoptosis Detection Kit, Intergen Co., Purchase, NY). The number of apoptotic hepatocytes were counted in 10 high power fields using a Zeiss Axiolab microscope (Carl Zeiss, Jena, Germany). This area of 10 high power fields (1.96 mm^2) contained 4,000 hepatocytes on average. Apoptotic cells were identified by morphologic criteria (cell shrinkage, chromatin condensation and margination, formation of apoptotic bodies) and by staining with the TUNEL assay according to the manufacturer's instructions (ApopTag Peroxidase In Situ Apoptosis Detection Kit, Intergen Co., Purchase, NY). Morphologic criteria of necrosis such as cell swelling, cell disruption, karyolysis and loss of architecture were evaluated in serial HE- stained sections. All histologic evaluations were done in a blinded fashion.

Transmission Electron Microscopy

At the end of the experiments, perfusion-fixation of hepatic tissue was performed using a 5% glutaraldehyde / 4% paraformaldehyde mixture in phosphate buffer (pH 7.4) via the hepatic artery at a constant pressure similar to normal mean aortic pressure of the rat. Specimens cut from the left liver lobe, were postfixed with osmium tetroxide, dehydrated in graded alcohol and embedded in Epon 812. Ultrathin sections were cut and contrasted with uranyl acetate and lead citrate for electron microscopy.[35]

Analytical Methods

GSH and GSSG

Total soluble glutathione (GSH and GSSG) was measured in plasma and in the acidic homogenate from freeze-clamped livers as described in detail elsewhere.[38] For GSSG analysis an aliqot (200 μL) of blood was mixed immediately with 200 μL of 10 mM N- ethylmaleimide (NEM) in 100 mM phosphate buffer (pH 6.5) containing 17.5 mM EDTA.[38] The remaining blood (300 μL) was centrifuged at full speed for 1 minute. An aliquot (100 μL) of plasma was pipetted into 100 μL sulfosalicylic acid (5%) for determination of total glutathione. To separate GSSG from NEM and NEM-GSH adducts, an aliquot of NEM treated plasma was passed through a Sep-PakC_{18} cartridge (Waters, Framingham, Ma, USA) followed by 1 mL of 100 mM phosphate buffer (pH 7.5). GSSG in the eluates and total glutathione in plasma and acidic homogenates were determined by an enzymatic test as described previously.[38,39] GSH plasma concentrations were calculated as the difference between total glutathione and GSSG.

Calpain Assay

Calpain protease activity was measured according to a modified method by Sindram et al.[21,40] 100 mg of tissue obtained from the left liver lobe after 2 hours of reperfusion were frozen immediately and stored at -80°C. 40 μL of diluted (1:10) cytosol extract was added to 160 μL of 50 μM Suc-Leu-Leu-Val-Tyr-7-amino-4-methyl coumarin (Sigma) dissolved in dimethyl sulfoxide and buffer containing 100 mM Tris-HCl and 145 mM NaCl at pH 7.3 in the presence or absence of 10 mM $CaCl_2$ at 37°C, following amino-4- methyl coumarin (AMC) release by fluorometry.[21,40] Protein concentration of the cell lysate was determined with Biorad-Bradford reagent and bovine serum albumin as standard. Calpain activity was expressed as pmol AMC release per mg protein per min at 10 mM $CaCl_2$.

Serum Aminotransferases

Serum aminotransferases were used as established markers of hepatic injury. Aspartarte aminotransferase (AST) and alanine aminotransferase (ALT) were measured 2 hours after reperfusion using a serum multiple analyzer (Hitachi 917, Roche Germany).

Statistical Analysis

All data are expressed as mean and standard error. Statistical differences between groups were calculated using paired or unpaired Student's t test for randomly distributed data and the Mann-Whitney U test for nonparametric data following analysis of variance (ANOVA). Comparison between survival groups was performed by the χ^2 test. Differences were considered significant at $P < 0.05$.

RESULTS

Effect of Intravenous GSH Administration on Hepatocellular Injury After Warm Ischemia

Substantial parenchymal cell damage following 60 and 90 minutes of normothermic ischemia was indicated by a more than 7-fold and almost 20-fold increase of serum transaminases, respectively (Fig. 1). Intravenous administration of 200 μmol GSH/h/kg significantly ($P < 0.05$) reduced ALT- (Fig. 1A) and AST- release (Fig. 1B) in both ischemic groups indicating protection against parenchymal liver cell injury. A similar protection was observed when animals were treated with 50 or 100 μmol GSH/h/kg.

Reduced parenchymal cell damage is supported by analysis of transmission electron microphotographs of livers subjected to 60 minutes of ischemia and 2 hours of reperfusion. Normothermic ischemia resulted in appearance of large vacuoles within hepatocytes (Fig. 2A). Furthermore, hepato-

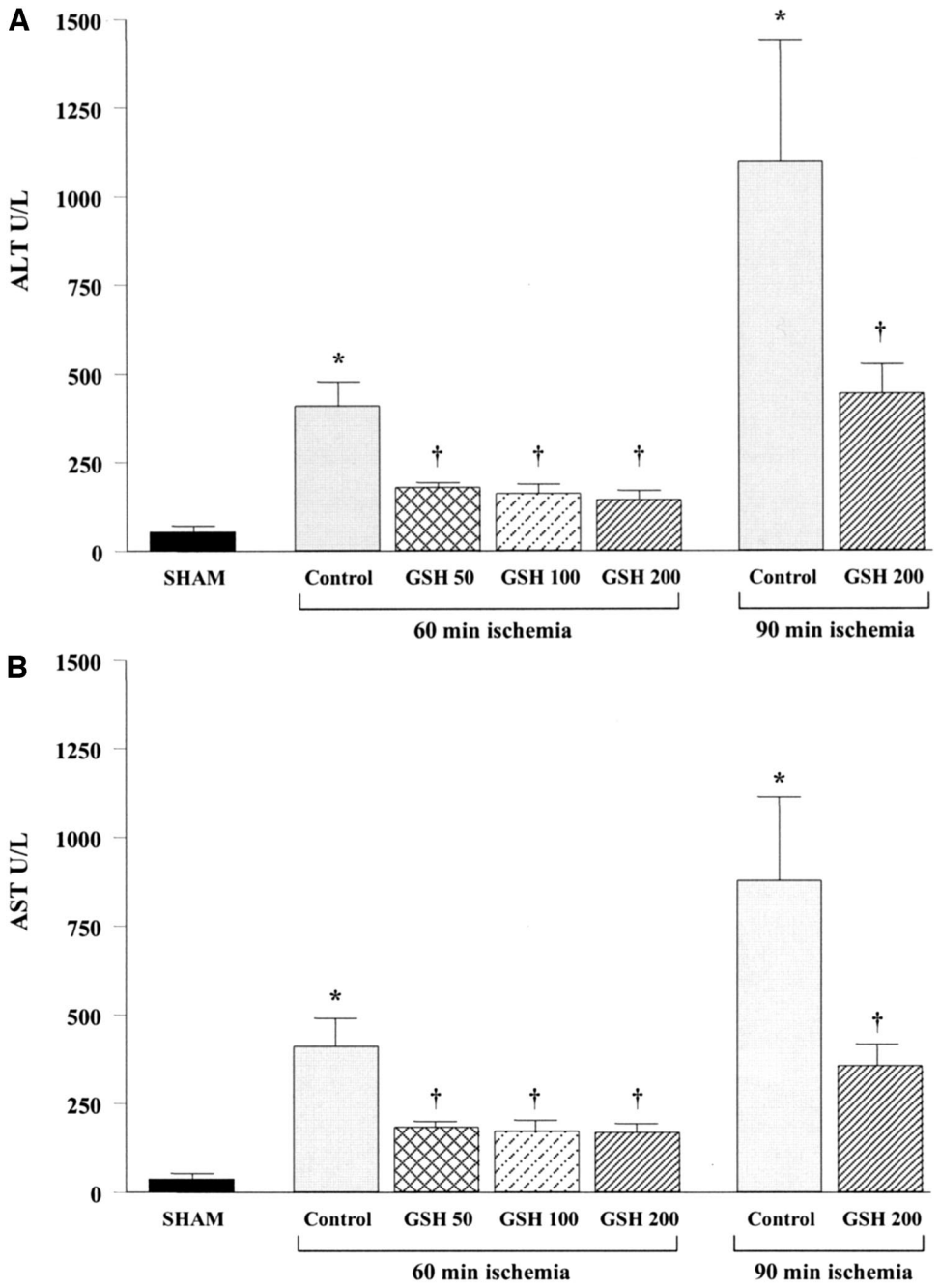

FIGURE 1. Effect of intravenous GSH treatment on serum ALT and AST plasma levels 2 hours after reperfusion of livers subjected to 60 or 90 minutes of partial warm ischemia. Intravenous infusion of 50 (n=8), 100 (n=8) or 200 (n=6) μmol GSH/h/kg was started 20 minutes before reperfusion and continued until the end of the 2 hours reperfusion period. Animals receiving GSH had significantly lower ALT (A) and AST (B) levels than untreated controls (n=8). Data are given as mean ± SEM; * $P < 0.001$ versus sham group (n=6); † $P < 0.05$ versus saline-treated controls.

cytes showed evidence for peroxisomal proliferation (Fig. 2A). Intravenous GSH- treatment markedly reduced the number of vacuoles and peroxisomes (Fig. 2B). No evidence for relevant damage to sinusoidal endothelial cells (SEC) could be identified in untreated or GSH- treated livers. Accordingly, HE stained sections of livers from rats treated with 100 μmol GSH/h×kg had a normal architecture and no sign of liver cell injury or relevant leukocyte accumulation (Fig. 2C).

Histologic sections of these livers were then evaluated for apoptotic cells using morphologic criteria (cell shrinkage,

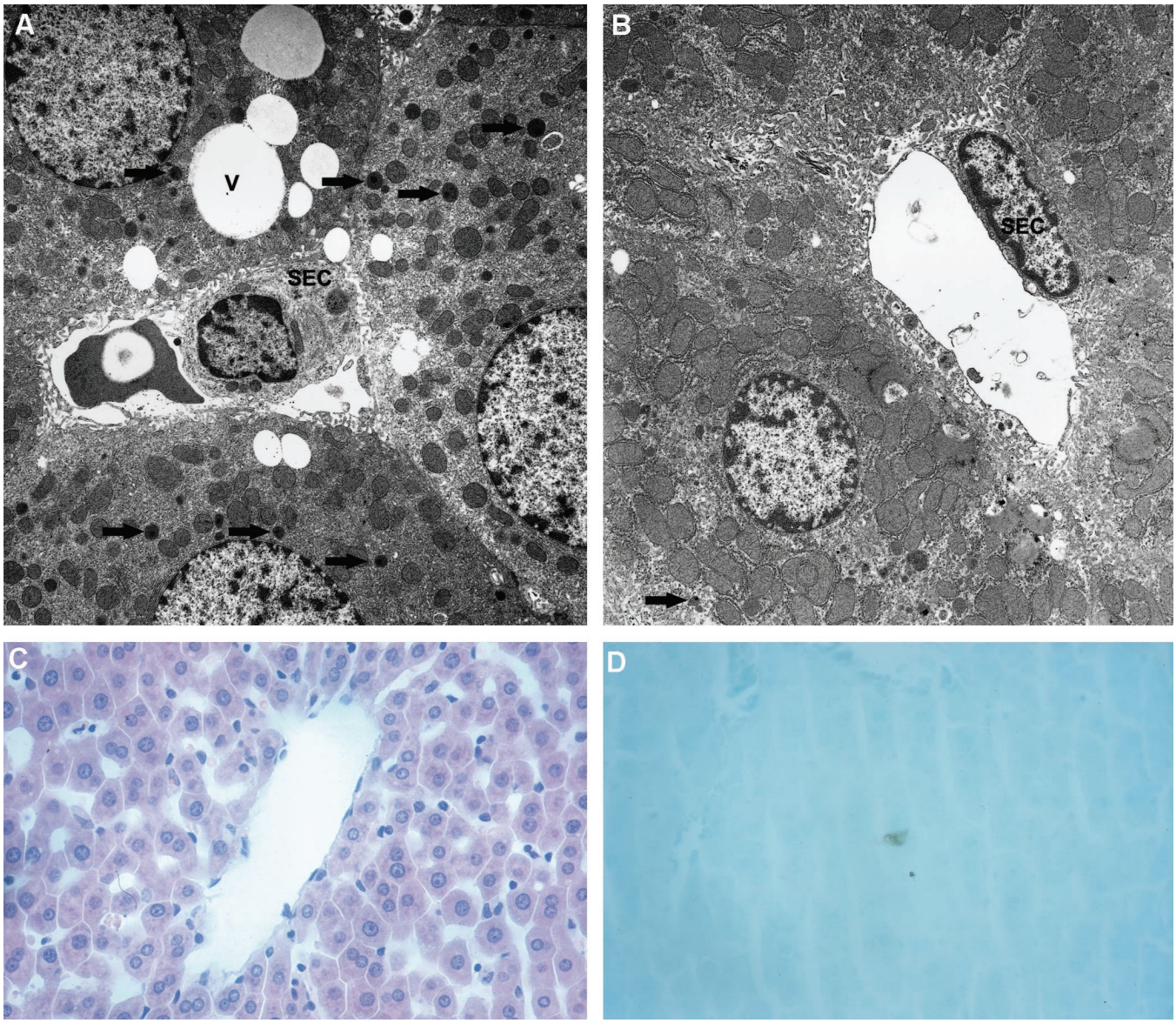

FIGURE 2. Representative electron micrographs, HE staining and TUNEL staining of livers subjected to 60 minutes of ischemia and 2 hours of reperfusion. (A) Hepatocytes of untreated livers showing considerable vacuolization (v) and peroxysomal proliferation (arrows). (B) Vacuolization and peroxisomal proliferation were absent in hepatocytes when animals received 100 μmol GSH/h/kg (x 3000). (C) HE stained liver sections from GSH- treated animals appeared normal with no change in the architecture. (D) Only rare hepatocytes stained TUNEL positive in untreated (not shown) and GSH- treated livers.

nuclear condensation and/or margination and the formation of apoptotic bodies) together with the TUNEL assay to support morphologic findings. At 2 hours of reperfusion following 1 hour of ischemia, apoptotic hepatocytes were rare (0.50 $\pm$ 0.10% of hepatocytes in 10 high power fields). Comparable small numbers of apoptotic hepatocytes were found in livers from animals treated with 100 μmol GSH/h$\times$kg upon reperfusion for 2 hours (0.65 $\pm$ 0.20%) (Fig. 2D) as well as in the nonischemic right liver lobes of untreated (0.50 $\pm$ 0.10%) and GSH- treated animals (0.60 $\pm$ 0.15%).

Effect of GSH Treatment on Animal Survival

A recently described model of segmental hepatic ischemia (70%) followed by resection of the nonischemic liver lobes (30%) at the end of ischemia was used to evaluate the

effects of postischemic GSH treatment on animal survival. After a 2-hour period of ischemia, a significant effect on long-term survival was observed in animals subjected to 200 μmol GSH/h/kg during the initial 2-hour reperfusion period. Six of 9 animals survived permanently (>30 days) whereas only 3 of 9 animals survived longer than 3 days of surgery in the control group ($P < 0.05$).

Effect of GSH Treatment on the Hepatic Microcirculation

Compared with sham-operated animals substantial disturbances of the hepatic microcirculation were observed in ischemic controls (Fig. 3). Thirty minutes after starting reperfusion approximately 20% of sinusoids were not perfused in both ischemic groups (Fig. 3A). Infusion of 200 GSH nearly completely prevented no-reflow of sinusoids after 60 or 90 minutes of ischemia (Fig. 3A). Intravital microscopy revealed considerable leukocyte sticking within sinusoids (Fig. 3B) and postsinusoidal venules (Fig. 3C). Administration of GSH reduced the number of permanently attached leukocytes to sinusoids as well as to postsinusoidal venules in a dose-dependent fashion (Fig. 3B,C). When control livers were exposed to 90 minutes of ischemia the number of leukocytes stagnant to sinusoids increased only minimally (Fig. 3B) whereas the number of leukocytes sticking to the endothelium of postsinusoidal venules was more than doubled (Fig. 3C). Again, treatment with 200 GSH significantly counteracted the adherence of leukocytes to sinusoids ($P < 0.001$) and postsinusoidal venules ($P < 0.01$) (Fig. 3B,C). Furthermore, GSH treatment reduced leukocyte rolling following 90 minutes of ischemia by more than 50% ($P < 0.001$) (Fig. 3D).

Effect of GSH Administration on Plasma GSH and GSSG Concentrations and the Intracellular Content of Total Glutathione

Intravenous administration of GSH increased plasma-GSH levels in a dose- dependent fashion. Maximum concentrations were reached at 2 hours of reperfusion (Table 1, Fig. 4), indicating a substantial improvement of the extracellular antioxidant capacity during reperfusion. Parallel to GSH concentrations, plasma GSSG-levels increased up to 5-fold reaching maximum concentrations 15 minutes after starting reperfusion (Fig. 4). Because untreated animals showed no relevant increase in plasma GSSG these findings suggest oxidation of intravenously applied GSH due to ROS detoxification. Despite the substantial increase of plasma GSH intracellular contents of total glutathione remained unchanged (Table 1).

Calpain Activity in Untreated and GSH-Treated Livers

Calpain activity was compared in the cytosol from livers subjected to 0 (Sham-operated), 60 or 90 minutes of ischemia (Fig. 5). Compared with normal liver, calpain activity increased significantly ($P < 0.05$) after 2 hours of reperfusion, but no significant difference was seen between the 60 and the 90 minutes ischemic groups. The increase of calpain activity following 60 minutes of ischemia was not influenced by the administration of GSH (Fig. 5). Administration of 200 μmol GSH / h / kg slightly decreased calpain activity after 90 minutes of ischemia (Fig. 5).

DISCUSSION

There is substantial experimental evidence demonstrating the impact of Kupffer cell- derived ROS on reperfusion injury of the liver following warm or cold ischemia.[8,12] Therefore, antioxidative strategies directed towards the vascular oxidant stress produced by activated KC might effectively prevent reperfusion injury. This contention is strongly supported by our study. We obtained the following main results: (1) Intravenous infusion of GSH during reperfusion causes a significant reduction of parenchymal cell injury as well as of microvascular reperfusion injury in livers subjected to prolonged warm ischemia; (2) Reduction of necrotic cell damage appears to be the principal mechanism of cytoprotection. GSH treatment markedly reduces the postischemic increase in plasma AST and ALT levels whereas apoptotic hepatocytes are rare in untreated and GSH- treated animals. (3) Postischemic GSH treatment is able to significantly improve animal survival; (4) Administration of GSH does not affect the intracellular GSH status but improves the vascular antioxidant capacity thereby enhancing ROS detoxification in extracellular compartments; (5) GSH confers protection without influencing the increase in calpain activity.

Prevention of Reperfusion Injury by Postischemic GSH Treatment

Reperfusion of rat livers subjected to 60 or 90 minutes of warm ischemia resulted in a 7-20-fold increase of plasma ALT and AST activities, indicating considerable parenchymal cell damage. Intravenous administration of GSH at doses of 50–200 μmol/h/kg significantly reduced ALT and AST release by approximately 50–60%. Because postischemic GSH treatment can only protect cells that are not already seriously damaged before the onset of reperfusion, our results clearly demonstrate prevention of reperfusion injury to parenchymal liver cells.

During the last years an increasing number of publications reported evidence for apoptotic cell death following warm or cold ischemia.[41–45] According to these studies 40–85% of hepatocytes undergo apoptosis during reperfusion. The quantification of apoptosis was mainly based on TUNEL assay. However, it is well known that the TUNEL method stains necrotic as well as apoptotic cells and, moreover, that morphology defines apoptosis and is the gold standard. Interestingly, if morphologic criteria are applied,

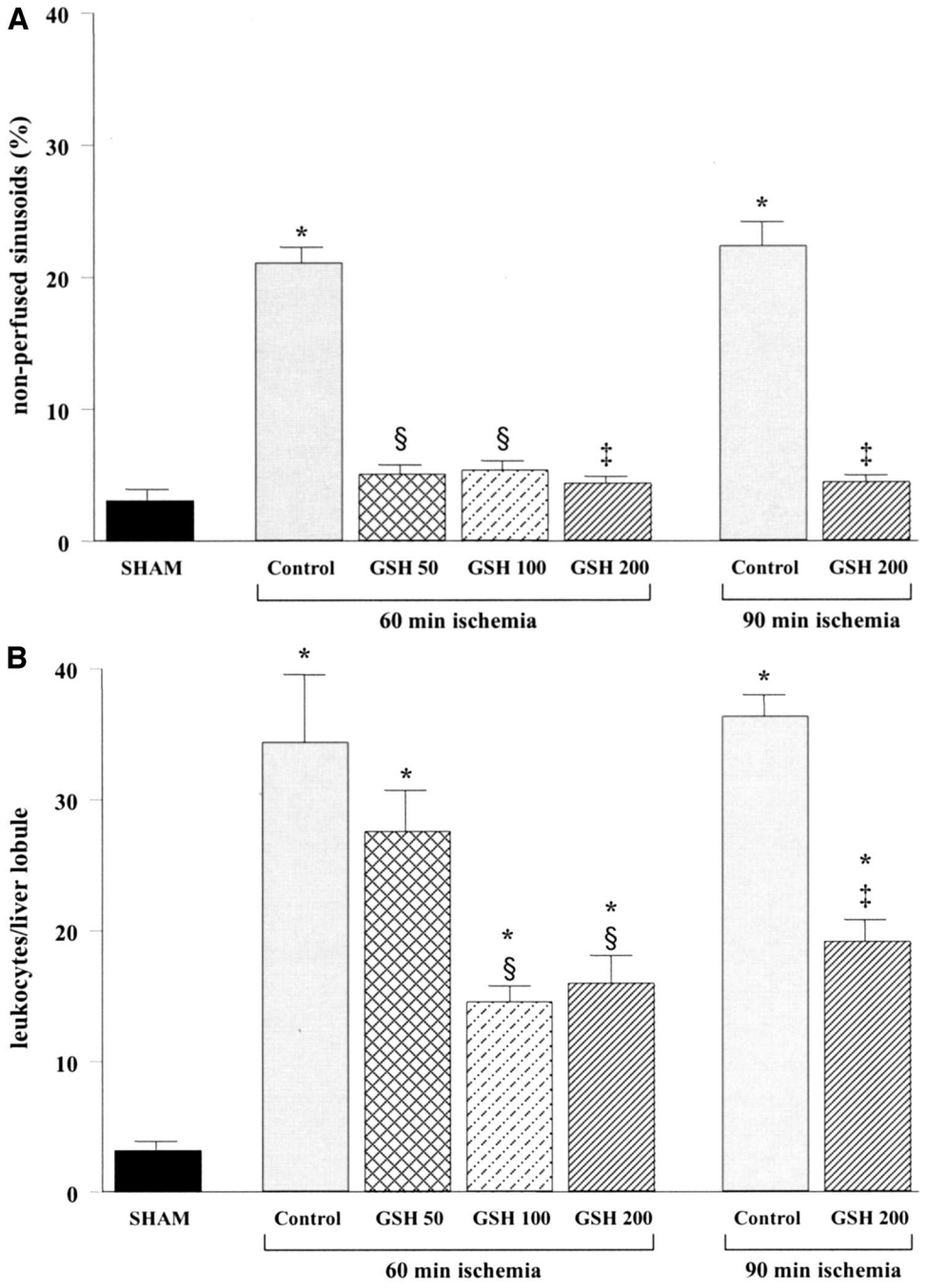

FIGURE 3. *Figure and Legend continue.*

only less than 1% of hepatocytes were found to undergo apoptosis following 1 hour of warm ischemia and 1, 6 and 24 hours of reperfusion.[46,47] Using TUNEL assay in combination with morphologic criteria we found a similar rate of apoptotic liver cells (0.5%) after 1 hour of ischemia and 2 hours of reperfusion which was not influenced by GSH treatment. These findings are in close agreement with recent studies[47] and suggest that prevention of necrosis, but not apoptosis, appears to be the principal mechanism of GSH-mediated hepatoprotection. The term necrosis refers to secondary changes after cell death which becomes histologically apparent after 6 hours of reperfusion.[47] Consequently, we found no signs of coagulative necrosis in HE stained liver tissues obtained after 2 hours of reperfusion. In contrast,

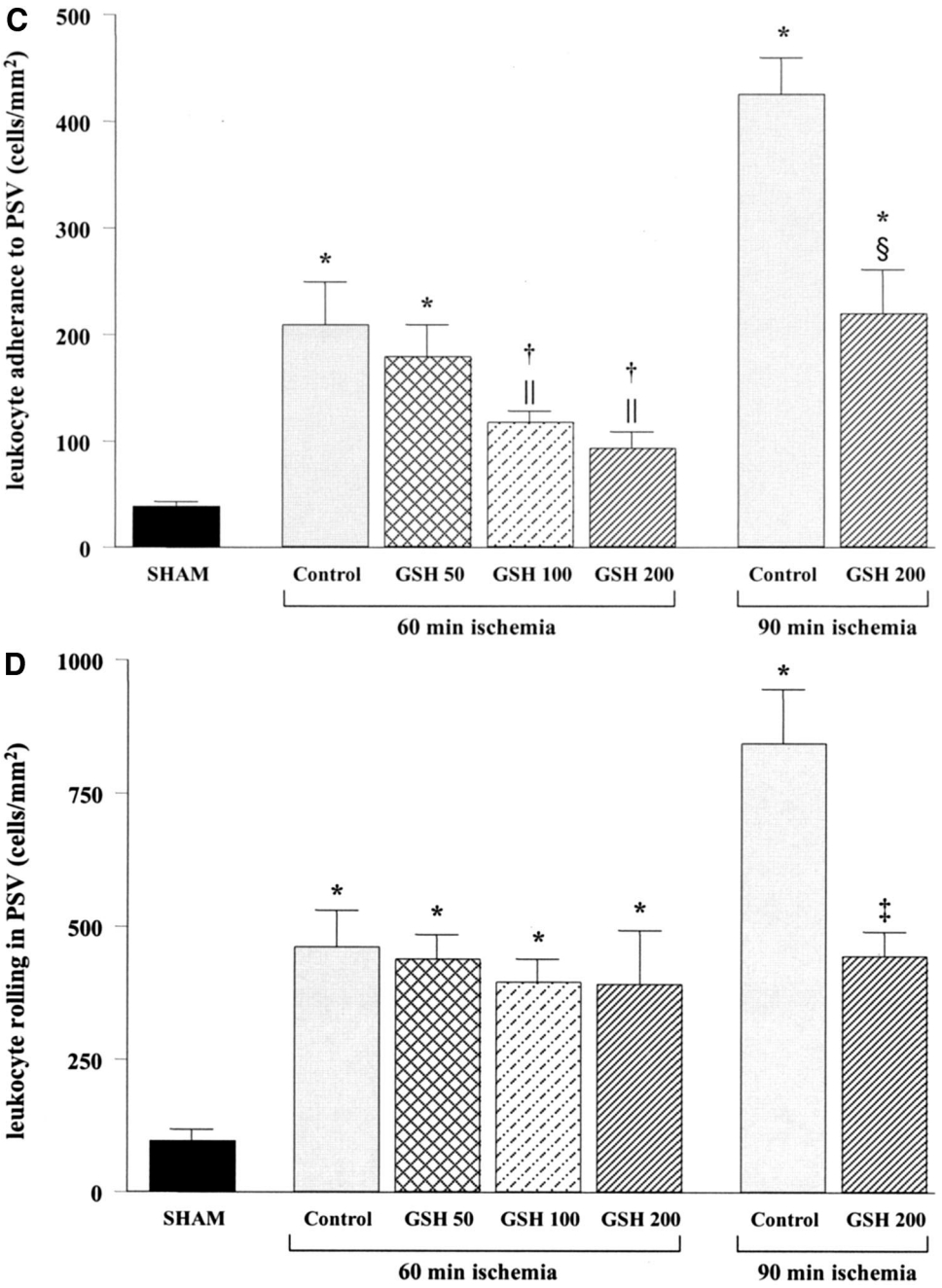

FIGURE 3. Impact of intravenous GSH treatment on postischemic disturbances of the hepatic microcirculation. IVM data were obtained 30 minutes after starting reperfusion of livers which underwent either 60 or 90 minutes of normothermic ischemia. Intervention groups receiving 50 (n=8), 100 (n=8) or 200 (n=6) μmol GSH/h/kg were compared with saline-treated controls (n=8) and sham- operated animals (n=6), respectively. (A) Nonperfused sinusoids; (B) permanent adherence (sticking) of leukocytes to sinusoids; (C) permanent adherence (sticking) of leukocytes to the endothelium of postsinusoidal venules (PSV); (D) temporary adherence (rolling) of leukocytes in postsinusoidal venules (PSV). Microhemodynamic parameters were analyzed in a blinded fashion by scanning a region of 10 randomly selected acinar areas and postsinusoidal venules. Data are given as mean ± SEM; * $P < 0.001$, † $P < 0.01$ versus sham group; ‡ $P < 0.001$, § $P < 0.01$, ‖ $P < 0.05$ versus saline- treated controls.

TABLE 1. Plasma GSH and Total Intracellular Glutathione After Warm Hepatic Ischemia

	Plasma GSH [μM]	Total intracellular GSH [μmol/g liver]
Sham	9.4 ± 0.3	3.32 ± 0.44
60 min of ischemia		
Control	10.5 ± 1.1	3.50 ± 0.39
GSH 50	103.9 ± 12.6*	4.19 ± 0.49
GSH 100	118.4 ± 13.3*	3.32 ± 0.26
GSH 200	299.1 ± 26.3*	4.24 ± 0.43
90 min of ischemia		
Control	8.9 ± 0.8	3.19 ± 0.38
GSH 200	277.8 ± 22.3*	3.65 ± 0.18

Note: Plasma GSH and the content of total glutathione (GSH and GSSG) in liver tissue was estimated 2 hours after reperfusion of livers subjected to 60 or 90 minutes of warm ischemia. GSH was intravenously applied at 50 (n = 8), 100 (n = 8) and 200 (n = 6) μmol/h/kg. GSH infusion was started 20 minutes before reperfusion and continued until the end of the 2 hours reperfusion period. Note: Values are mean ± SEM. *p < 0.001 versus sham-operated animals or saline-treated controls.

electron microscopy analysis showed a relevant vacuolization within hepatocytes, a generally accepted morphologic criterion of necrosis. GSH treatment prevented vacuolization, which gives further support for an antinecrotic mechanism of GSH action.

The functional relevance of these findings was confirmed by a doubling of animal survival rate. Postischemic GSH treatment can therefore be considered as a powerful protective strategy able to convert rapid animal death after a prolonged duration of ischemia to consistent long-term survival. Based on GSH plasma levels following GSH infusion, concentrations between 100 and 300 μM GSH seems to be pivotal for cytoprotection. Since plasma GSH levels up to 500 μM showed no toxicity in humans[22] GSH seems as ideal and presumably safe candidate drug for prevention of reperfusion injury of the liver

Mechanisms of Cytoprotection by GSH

Previous studies showed that the spontaneous oxidation of intravenously administered GSH is a relatively slow process.[8] Our experiments support this contention. When animals were treated with 200μmol GSH/h/kg plasma GSSG declined from maximal levels of 6 μM at 15 minutes to 3.5

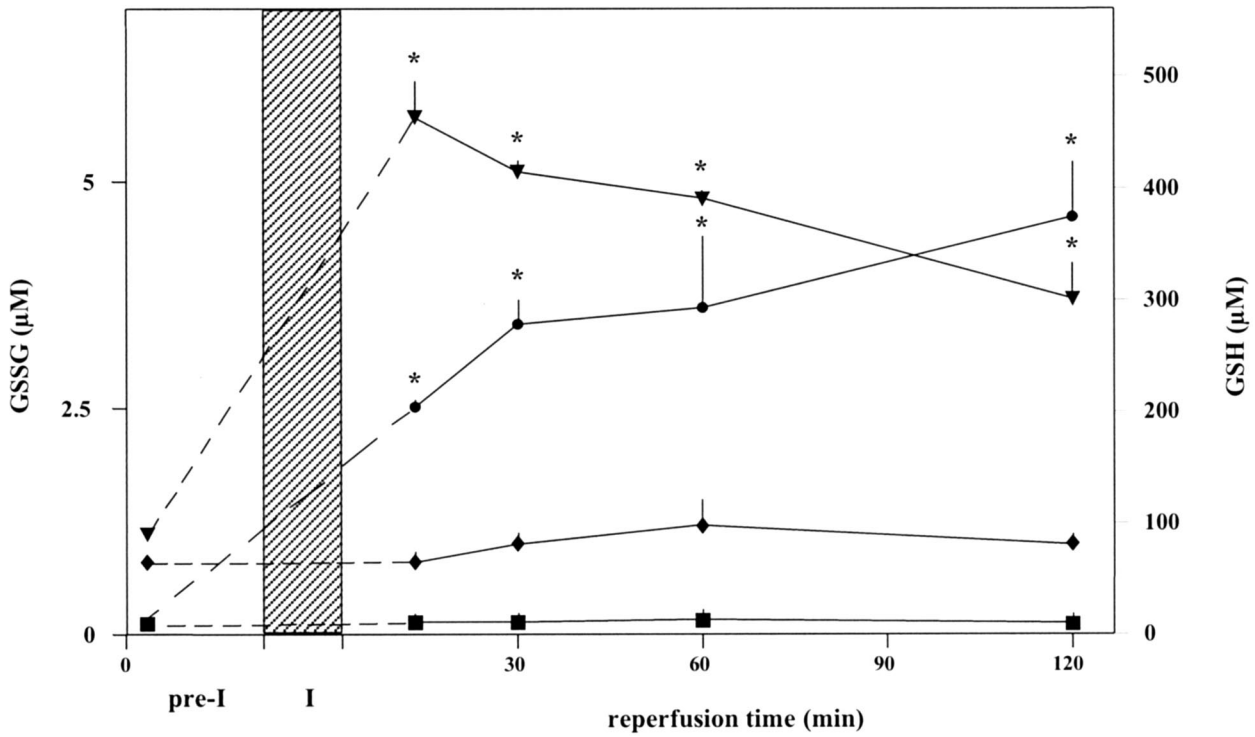

FIGURE 4. Effect of GSH treatment on plasma GSH and GSSG levels following 90 minutes of warm hepatic ischemia. Following reperfusion of control livers (n=8) plasma GSH (▶) and GSSG (♦) remained unchanged. Intravenous infusion of 200 μmol GSH/h/kg resulted in a continuous increase of plasma GSH (●) until the end of reperfusion (n=6). In contrast, plasma GSSG (▶) arised to maximum levels after 15 minutes but thereafter declined continuously. Data are given as mean ± SEM; * $P < 0.01$ compared with saline- treated controls.

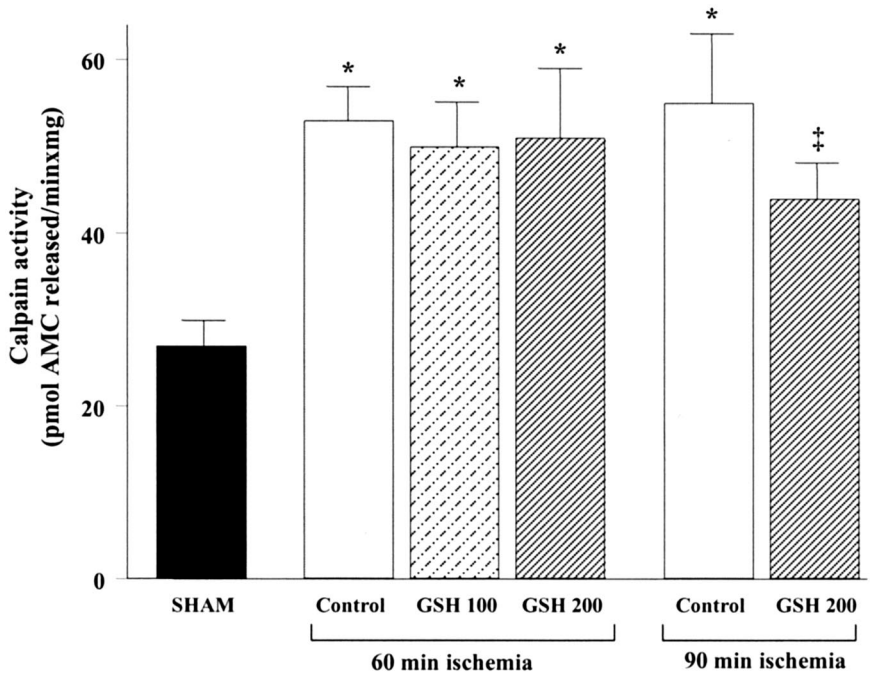

FIGURE 5. Cytosolic calpain activity after reperfusion in livers subjected to 60 or 90 minutes of warm ischemia. When livers were subjected to 60 minutes of ischemia calpain activity increased significantly after 2 hours of reperfusion ($P < 0.001$) but was not influenced by intravenous GSH treatment. Following 90 minutes of ischemia there was a slight decrease of calpain activity in GSH treated livers. Data are given as mean ± SEM; * $P < 0.001$ and ‡ $P < 0.01$ versus sham group.

μM at 2 hours of reperfusion whereas plasma GSH raised from 200 to 400 μM, respectively. Consequently, the observed increase of plasma GSSG cannot be explained by autoxidation of GSH in blood but reflects an accelerated oxidation of GSH during early reperfusion. Because intracellular formation of GSSG is low in reperfused livers[48] increased plasma GSSG formation is likely to take place extracellularly. These findings strongly suggest intravenously applied GSH as a direct or indirect antioxidant against ROS derived from activated KC or leukocytes.

Earlier animal studies demonstrated prevention of hepatic ischemia-reperfusion injury by N-acetycysteine (NAC),[27] a precursor of the natural antioxidant glutathione (GSH). However, as a main result of the present study NAC and GSH seem to protect the liver by rather different mechanisms. NAC is rapidly taken up by liver cells and used for the synthesis of GSH. Accordingly, prevention of ischemia-reperfusion injury by NAC was attributed to an increase in intracellular GSH. In contrast, the present study demonstrated that intravenous application of GSH upon reperfusion results in a marked increase of extracellular (plasma) GSH, but not intracellular GSH. These findings indicate that a powerful protection can be achieved by direct application of GSH thereby increasing the extracellular, but not the intracellular antioxidant capacity. Furthermore, the half life of GSH is very short (2 minutes).[49] Accordingly, elevated plasma GSH levels achieved in our study returned to baseline within 10–15 minutes after termination of GSH infusion. Thus, a relevant accumulation of GSH exceeding the initial 2 hours reperfusion period can be ruled out in the present study. Nevertheless, animal survival was markedly improved. These findings strongly suggest that reduction of *early* reperfusion injury by an *extracellular* antioxidative intervention acting exclusively during the first 2 hours of reperfusion translates to an improved outcome in vivo.

GSH remarkably improved the hepatic microcirculation in a dose-dependent fashion. In particular, the number of nonperfused sinusoids decreased to that observed in nonischemic livers.[34] Moreover, GSH administration significantly decreased the number of leukocytes permanently sticking to sinusoids as well as to postisinusoidal venules. In accordance to the proposed stepwise process of leukocyte-endothelial interaction from leukocyte rolling as initial event to permanent attachment of leukocytes as the second step,[50] our results suggest ROS as a mediator of both steps: treatment with GSH prevented leukocyte sticking and rolling in postsinusoidal venules of livers subjected to 90 minutes of ischemia. The same effects were observed during transplantation of KC-depleted rat livers.[51] Thus, our findings provide further evidence for a close relationship between KC- derived ROS and the development of hepatic microcirculatory failure upon reperfusion.

Recently, it has been shown that calpains play an important role in mediating necrotic and apoptotic cell death of hepatocytes and SEC after warm or cold ischemia.[20,21] In agreement with these studies we found a 2- fold increase of calpain activity in liver tissue after 2 hours of reperfusion. Increased calpain activity has generally been related to increased cytosolic calcium concentrations during ischemia.[52] During reperfusion, generation of ROS can contribute to further increases in calpain activity.[19] In this study, we found no effects of GSH treatment on calpain activity. Therefore, it seems unlikely that GSH treatment influences mechanisms involved in the process of calpain activation. Assuming that intravenously applied GSH traps ROS released by Kupffer cells into sinusoids our findings argue against vascular oxidant stress as a causal factor of calpain activation.

In summary, the present study demonstrates prevention of reperfusion injury in the rat liver after prolonged warm ischemia by postischemic intravenous administration of GSH. Treatment with GSH prevented microcirculatory failure and injury to hepatocytes and, moreover, improved animal survival. Protection was associated with an increased formation of plasma GSSG providing evidence for an accelerated detoxification of ROS by intravenously applied GSH. Therefore, intravenous administration of GSH appears to be a candidate therapy for the prevention of reperfusion injury of the liver after prolonged warm ischemia. Because GSH is well tolerable also in man, this novel approach could be introduced to human liver surgery.

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